AXL inhibition sensitizes mesenchymal cancer cells to anti-mitotic drugs

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Precis: Findings challenge a purported role for AXL in drug resistance while offering a novel rationale to combine AXL targeting drugs with anti-mitotic agents to eradicate invasive cancers.

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ABSTRACT

Molecularly-targeted drug therapies have revolutionized cancer treatment, however, resistance remains a major limitation to their overall efficacy. Epithelial to mesenchymal transition (EMT) has been linked to acquired resistance to tyrosine kinase inhibitors (TKIs), independent of mutational resistance mechanisms. AXL is a receptor tyrosine kinase (RTK) associated with EMT that has been implicated in drug resistance, and has emerged as a candidate therapeutic target. Across 643 human cancer cell lines that were analyzed, elevated AXL was strongly associated with a mesenchymal phenotype, particularly in triple negative breast cancer and non-small cell lung cancer. In an unbiased screen of small molecule inhibitors of cancer-relevant processes, we discovered that AXL inhibition was specifically synergistic with anti-mitotic agents in killing cancer cells that had undergone EMT and demonstrated associated TKI resistance. However, we did not find that AXL inhibition alone could overcome acquired resistance to EGFR TKIs in the EMT setting, as previously reported. These findings reveal a novel co-treatment strategy for tumors displaying mesenchymal features that otherwise render them treatment-refractory.
INTRODUCTION

Epithelial to mesenchymal transition (EMT) is a vital cellular process during normal development, and contributes to the invasive and metastatic properties of human tumors (1). EMT has also been implicated in resistance to multiple cancer drug therapies, including several tyrosine kinase inhibitors (TKIs). Thus, drug-sensitive cancer cell lines selected in culture for acquired drug resistance can adopt a mesenchymal phenotype (2-4). Moreover, EMT has been associated with acquired resistance to Epidermal Growth Factor Receptor (EGFR) TKIs in EGFR mutant lung cancer patients (2, 5).

The receptor tyrosine kinase AXL is a member of the TAM family kinases, which also includes TYRO-3 and MER (6). AXL is overexpressed in many solid tumors (4, 7-10), although, activating mutations have not been observed (11). AXL has one known ligand, Gas6 (12), and ligand binding promotes cell proliferation, survival and migration through activation of the PI-3K/AKT/S6K and ERK/MAPK pathways (9, 13-15). AXL expression may be a negative prognostic factor for breast and pancreatic cancer patients (7, 9), and may be a unique EMT effector in breast cancer progression (7, 16).

The association of AXL with EMT has prompted interest in AXL as a therapeutic target. AXL has been associated with a mesenchymal signature in non-small cell lung cancer (NSCLC) and has been proposed as a therapeutic target in EGFR TKI resistance (17), and in a model of acquired erlotinib resistance, EMT associated
with elevated AXL appeared to underlie erlotinib resistance (4). Moreover, co-
treatment of EGFR wild-type NSCLC xenografts with erlotinib and an anti-AXL
antibody decreased tumor volume and metastasis (18). Similarly, lapatinib-
resistant HER2-positive breast cancer cells demonstrated elevated AXL (19), and
AXL knockdown reportedly re-sensitizes imatinib-resistant CML cell lines to
imatinib (20).

We broadly surveyed AXL expression in a large panel of human cancer cell lines,
and assessed its role in drug resistance associated with a mesenchymal
phenotype. AXL expression was well correlated with a mesenchymal phenotype in
the context of both intrinsic and drug resistance-associated EMT. AXL inhibition did
not detectably increase sensitivity to TKIs as previously reported; however, we
observed a striking synergistic interaction between AXL inhibition and anti-mitotic
agents specifically in mesenchymal tumor cells.

MATERIALS AND METHODS

Cell lines
Cell lines were from American Type Culture Collection or Deutsche Sammlung von
Mikroorganismenund Zelkulturen, and were obtained between 2010 and 2013. All
cell lines were authenticated by STR profiling and SNP fingerprinting, and were
confirmed to be mycoplasma-negative by the Genentech cell line banking facility
(gCELL) prior to use (see Supplementary Procedures). Cells were maintained in culture for a 6 week period and thereafter a fresh vial of cells was obtained from gCELL. To induce EMT, cells were treated with 2ng/ml of rh-TGF-β1 every three days over a 2-3 week period.

**Inhibitors**

Erlotinib and cisplatin were from LC laboratories. Docetaxel and doxorubicin were from Sigma. PF-03814735, BI-2536, MP-470 and PHA-739358, were from Selleck Chemicals. Paclitaxel was from Tocris. Gemcitabine was from Toronto Research. R428 was from Synkinase. Recombinant human AXL-Fc, Gas6 and TGF-β1 were from R&D Systems.

**RNA-seq analysis**

RNA from cell lines and was used to prepare libraries using the TruSeq RNA Sample Preparation kit (Illumina). Libraries were sequenced on the Illumina HiSeq 2000 platform. Expression of AXL and Gas6 RNA was extracted from a database derived from RNA-seq analysis [Klijn et al 2014, Manuscript in preparation].

**Immunoblotting**

Immunodetection of proteins was performed using standard protocols. The AXL, Mer, Tyro-3, E-Cadherin, vimentin, phospho-CDC2 (Tyr 15), CDC2, phospho-AKT, total AKT, phospho-S6, total S6, and GAPDH antibodies were from Cell Signaling Technology, and the PARP antibody was from eBioscience.
Gas6 ELISA

Cells were incubated overnight and replenished in fresh RPMI 1640 media. Medium was collected 24h later and was analyzed using the Human Gas6 Quantikine ELISA Kit from Quantikine, R&D systems. ELISA was performed as per manufacture’s instructions and normalized to cell number.

Invasion assay

Pre-labeled DilC12(3) BD Bioscience cell suspensions in serum-free RPMI media were added to the apical chamber of the BD BioCoat™ Tumor Invasion System, 8µm from BD Biosciences. Invasion assays were performed as per manufacturer’s instructions.

Kinase Profiling

The R428 kinase profile was performed at KINOMEscan. R428 was screened at 1000nM, and results for primary screen binding interactions are reported as percentage control, where lower numbers indicate stronger binding.

Cell Viability

Cell viability was assessed as previously described (21).

RNA interference
AXL knockdown was achieved by transfection using ON-TARGETplus AXL siRNA (Dharmacon) and Lipofectamine RNAiMax (Invitrogen). ON-TARGETplus Non-targeting Pool siRNA (Dharmacon) served as control.

**Time-lapse imaging**

Cells were seeded in a glass-bottom 24-well plate (Greiner Bio-One). 100 cells per treatment were tracked and mitotic fate and cell death were scored as described previously (22).

**Xenograft studies**

All procedures were approved by and conformed to the guidelines and principles of the Institutional Animal Care and Use Committee of Genentech and were carried out in an Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility. Five million HeLa or MDA-MB-231 cells were inoculated in the right flank of Nu/Nu nude mice. When tumors reached 100-200 mm$^3$, mice were treated with vehicle control, R428 (125 mg/kg five times per week; oral gavage), docetaxel (10 mg/kg three times a week, intravenously for HeLa and 10 mg/kg once a week, intravenously for MDA-MB-231), or the combination. Tumors were measured three times weekly using digital calipers and tumor volumes were calculated as $(L \times (W \times W))/2$. Differences between R428 and docetaxel combination groups and individual-treated and control groups were determined using two-way ANOVA.
Statistics

Bliss expectation was calculated as \((A + B) - A \times B\), where \(A\) and \(B\) are the fractional growth inhibitions of drug \(A\) and \(B\) at a given dose. The difference between Bliss expectation and observed growth inhibition of the combination of drugs \(A\) and \(B\) at the same dose is the "Delta Bliss excess." Delta Bliss values were summed across the dose matrix to generate the Bliss sum. Differences between two groups were determined using Student’s t-test, \(p\) values are represented as \(p<0.05\) *, \(p<0.01\) ** and \(p<0.001\) ***. Differences between the R428 and docetaxel combination groups and individual-treated and control groups were determined using two-way ANOVA.

RESULTS

AXL expression correlates with a mesenchymal phenotype

Using RNAseq, we profiled mRNA expression of \(AXL\) (Figure 1A and Supplementary Dataset S1) and its ligand \(Gas6\) (Figure S1A) in 643 human cancer cell lines. \(AXL\) expression in cell lines from breast and lung demonstrated the greatest dynamic range within a tissue subset, where expression levels could readily classify most lines as “\(AXL\)-high” or “\(AXL\)-low”. \(Gas6\) mRNA was more uniformly expressed. To identify a potential correlation between \(AXL\) expression and the mesenchymal phenotype, we examined expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin (Figure S1B). \(AXL\)-high cells generally expressed abundant vimentin whereas \(AXL\)-low cells demonstrated
higher E-cadherin expression. AXL-high cells showed, on average, a 4.6 fold increase in vimentin relative to AXL-low cells (p value = 2.04e-24) (Figure S1C). Thus, AXL expression is strongly associated with a mesenchymal phenotype in human cancer cell lines.

Within breast cancer subtypes, AXL is relatively elevated within the triple negative breast cancer (TNBC) subset compared to ER-positive/PR-negative and HER2 amplified subsets (Figure 1B). Gas6 is similarly elevated in the TNBC subset (Figure S1D). Correspondingly, AXL protein was highly elevated in TNBC relative to the other subtypes (Figure 1C). We expanded the TNBC panel and observed a particularly strong correlation between AXL expression and the mesenchymal subset of TNBC lines (Figure 1D, S1E). Expression of the AXL-related TYRO-3 RTK is variable, and is not correlated with any particular subset, and MER expression is inversely correlated with AXL (Figure 1D).

Immunohistochemistry analysis of TNBC tumors revealed that most expressed E-Cadherin, with 20/26 cases exhibiting strong membranous staining in all tumor cells and 4 displaying focal expression in malignant cells (Figure S2). Significantly, 7/26 samples displayed vimentin tumor cell staining in varying proportions of malignant cells (Figure S2). In all samples, vimentin and AXL were detected in normal stroma cells. AXL staining of a small proportion of tumor cells was seen in 3/26 tumor samples (Figure S2). The observed percentage of AXL-positive TNBCs is consistent with recent previous reports (23, 24).
In NSCLC, a previous report correlated the mesenchymal phenotype with sensitivity to the EGFR TKI, erlotinib (25). Indeed, AXL tended to be elevated in erlotinib-insensitive cells with relatively high vimentin (Figure 1E). Conversely, cell lines with greater erlotinib sensitivity tended to exhibit lower vimentin and AXL expression. This relationship was also observed with RNAseq analysis (Figure S1F). These results indicate a strong correlation between AXL expression and a mesenchymal/drug-resistant phenotype in breast cancer and NSCLC cells.

TGF-β-induced EMT is associated with increased AXL and TKI resistance

To explore a functional requirement for AXL in EMT-associated drug resistance, we experimentally induced EMT in cancer cell lines with previously established drug sensitivity by treatment with TGF-β (1, 26, 27). Exposure of EGFR- or HER2-“addicted” cell lines to TGF-β yielded the expected EMT, with transition from a compact uniform appearance to a spindle-like morphology (Figure 2A), as well as loss of E-cadherin and gain in vimentin (Figure 2B), and increased invasion capacity (Figure 2C). EMT is a reversible process, and indeed, upon withdrawal of TGF-β for 2 weeks, the mesenchymal cells reverted to an epithelial phenotype (Figure S3A).

Expression of AXL and Gas6 were substantially induced upon EMT, consistent with AXL’s association with a mesenchymal phenotype (Figure 2B,D). Significantly, HER2-amplified HCC1954 cells, following EMT, exhibited 10-fold reduced
sensitivity to the HER2/EGFR TKI lapatinib (Parental; IC\textsubscript{50}= 0.456\,\mu M, TGF-\beta; IC\textsubscript{50}= 4.036\,\mu M) (Figure 2E). Similarly, \textit{EGFR} mutant NSCLC PC9 cells exhibited 4-fold reduced erlotinib sensitivity following EMT, (Parental; IC\textsubscript{50}= 0.05\,\mu M, TGF-\beta; IC\textsubscript{50}= 0.214\,\mu M) (Figure 2F). In HCC1954 and PC9 cells, post-EMT, TKI treatment failed to promote apoptosis (Figure 2G). TKI resistance in the mesenchymal cells did not reflect drug efflux, as TKIs induced strong suppression of downstream signaling (Figure S3B). Thus, upon TGF-\beta-induced EMT in RTK oncogene-addicted cancer cells lines, the resulting mesenchymal cells exhibit substantially increased AXL, and become drug-resistant.

\textbf{EMT-associated drug resistance is independent of AXL function}

Recent reports have implicated AXL in acquired drug resistance (4, 19, 20). Therefore, we employed these models to examine a functional AXL requirement in EMT-associated drug resistance. Initially, we utilized R428, an AXL kinase inhibitor (Supplementary Dataset S2) (28). As expected, R428 effectively suppressed Gas6-induced phospho-AXL (Figure S4A), phospho-AKT and phospho-S6 levels (Figure S4B&C), and decreased invasion capacity of AXL-expressing cells (Figure S4D). These effects were not observed in AXL-negative BT-20 cells. We tested the ability of R428 to restore erlotinib sensitivity in a TGF-\beta-induced resistance model and in two models of acquired erlotinib resistance demonstrating EMT and increased AXL (Figure 3B and S5A). In both parental and drug-resistant cell lines we observed either very weak R428/erlotinib synergy (positive delta Bliss) or antagonistic interaction (negative delta bliss) (Figure 3A&C), with no change in cell
viability (Figure 3A). Similarly, there was no change in the IC$_{50}$ value for erlotinib sensitivity in combination with R428 (1µM) in either model (Figure 3D, E). Furthermore, AXL knockdown did not restore erlotinib sensitivity in either of the resistance models (Figure 3F-H, S5B). In addition, inhibition of AXL using an AXL-Fc fusion protein, an anti-AXL antibody, YW327.6S2 (Figure S5C & D), doxycycline-inducible AXL knockdown (Figure S5E-G), or overexpression of AXL (Figure S6A-C), does not detectably impact sensitivity to erlotinib. Finally, cotreatment of resistant cells with either AXL-Fc fusion protein or the AXL antibody with erlotinib did not prevent the emergence of drug-resistant clones (Figure S6D).

We next examined R428 activity in an AXL-negative EMT-associated resistance model. EGFR mutant HCC827 NSCLC cells, upon TGF-β treatment, become mesenchymal (Figure 1E, lane 48 and 49) and erlotinib-resistant (Figure 3D), but do not exhibit AXL induction (Figure 1E). R428 did not sensitize these cells to erlotinib (Figure 3D). Furthermore, MP-470, another AXL inhibitor (4), did not synergize with erlotinib (Figure S7A). Notably, AXL expression was consistently suppressed by TKI treatment in the mesenchymal cells (Figure 2G), further indicating that AXL is not required for the observed resistance. These collective observations do not support a role for AXL inhibition in overcoming acquired resistance to TKIs in the EMT context.

**AXL inhibition sensitizes erlotinib-resistant mesenchymal cells to docetaxel**
TGF-β-induced EMT or erlotinib-induced resistance yields mesenchymal cells that are cross-resistant to many anti-cancer agents (Table S1 and data not shown). We explored whether AXL inhibition could sensitize resistant mesenchymal cells to other anti-cancer agents. PC9 cells and mesenchymal derivatives were screened for sensitivity to 100 anti-cancer agents. Mesenchymal cells were selectively resistant to many agents—most notably, to docetaxel, an anti-mitotic agent (Table S1). We extended this observation to other erlotinib resistance models. In both the erlotinib-induced resistance (ERL-R) and TGF-β-induced mesenchymal resistance (MES) models the mesenchymal cells were more docetaxel-resistant (Figure 4A). Co-treatment with docetaxel and R428 was synergistic in both models (Figure 4A, 4B). Similarly, in the drug-resistant HCC827 ERL-R and HCC4006 ERL-R cells, greater sum Bliss scores were observed (Figure 4B). In contrast, we did not observe synergy with R428 and gemcitabine (Figure 5A).

Co-treatment of drug-resistant mesenchymal cells with R428 and docetaxel dramatically shifted docetaxel IC$_{50}$ values. Thus, for mesenchymal PC9 cells, the IC$_{50}$ for docetaxel alone was >300nM while in combination with R428 it was 0.3nM (Figure 4C). Similarly, in the erlotinib-resistant mesenchymal HCC4006 cells, the IC$_{50}$ for docetaxel alone was >300nM whereas in combination with R428, it was 0.191nM (Figure 4D). Conversely, in mesenchymal HCC827 cells, R428 did not synergistically interact with docetaxel, suggesting that the observed synergy reflects an AXL-targeted effect of R428 (Figure 4C). We observed similar synergy between the AXL inhibitor MP-470 and docetaxel (Figure S7A) and no change in
IC$_{50}$ values upon co-treatment with MP-470 and docetaxel in the AXL-negative mesenchymal HCC827 cells (Figure S7B). Collectively, these findings suggest that EGFR mutant cancer cells that become resistant to erlotinib and cross-resistant to docetaxel through innate or drug-induced EMT, can be sensitized to docetaxel by AXL inhibition.

**AXL inhibition sensitizes mesenchymal cancer cells to anti-mitotic agents**

To establish that R428/docetaxel synergy is independent of TGF-β or drug exposure, we evaluated this treatment combination in a panel of 100 cancer cell lines (Supplementary Dataset S3). We observed strong synergy in a subset of AXL-expressing mesenchymal cell lines (Figure 5A and Supplementary Dataset S3), and a correlation between AXL expression and the sum Bliss score (Figure 5B). Similarly, R428 synergized with the related taxane paclitaxel to inhibit proliferation (Figure S8A-D), but not with gemcitabine, doxorubicin, or cisplatin (Figure 5A,C and S8F&G). We also observed similar synergy between docetaxel and an AXL-Fc fusion protein, which blocks Gas6 binding to AXL (18), or with MP-470, in AXL-high cell lines (Figure 5D and S7C), and siRNAs targeting AXL further confirmed that AXL inhibition mediated the observed synergy (Figure 5E).

Tubulin-binding taxanes cause a sustained mitotic block, resulting in cell death during mitosis (29). To determine whether R428 synergistically interacts with anti-mitotic agents with distinct mechanisms of action, we tested two aurora kinase inhibitors, PHA-739358 and PF-03814735, and the PLK1 (polo kinase) inhibitor BI-
2536, which activates the spindle checkpoint, causing mitotic arrest (Figure 5A). We observed synergy between R428 and the aurora kinase and PLK1 inhibitors in AXL-expressing mesenchymal cells (Figure 5A,C). We also observed a five-fold shift in the PHA-739358 IC$_{50}$ upon combination treatment with R428 for the MDA-MB-231 cells (PHA-739358 alone, IC$_{50}$ = 1.63µM and in combination with R428 (1µM), IC$_{50}$ = 0.34µM (Figure 5C). Thus, AXL inhibition synergistically blocks cell proliferation in combination with anti-mitotic agents in mesenchymal cancer cells.

**AXL kinase inhibition enhances docetaxel activity to suppress tumor growth**

We extended the cell line findings to an *in vivo* context using tumor xenografts. First, we determined that R428 was pharmacologically active in xenografts (Figure 6A). To assess efficacy, mice bearing 100-200mm$^3$ tumors were treated with R428 and/or docetaxel. R428 or docetaxel slightly inhibited tumor growth; however, co-treatment caused significant tumor growth suppression (Figure 6B). Similar effects were seen with MDA-MB-231 xenografts (Figure 6C). A modest decrease in body weight appeared to be docetaxel-dependent (Figure 6B, 6C). These observations support the potential *in vivo* utility of AXL inhibitors combined with anti-mitotic agents.

**AXL inhibition enhances cell death during mitosis when combined with anti-mitotic agents**

To establish whether AXL inhibition alters mitotic cell fate when combined with anti-mitotic agents, we employed time-lapse microscopy of individual cells to monitor
duration of mitosis and cell fate during mitosis (Figure 7A, S9 and Supplemental Movies 1-4). The duration of completed mitosis in DMSO-treated HeLa cells ranged from 20-50 minutes (Figure 7B), and R428 did not affect duration (Figure 7B and Supplemental Movie 1&2). By contrast, docetaxel caused mitotic slippage generating three or four daughter cells (Figure 7A and Supplemental Movie 3) and a longer duration of mitosis, as expected (Figure 7B). Upon co-treatment with docetaxel and R428, the percentage of cells that completed mitosis decreased substantially, and the percentage of cells that died in mitosis was greatly enhanced, from 46% to 93% (Figure 7A, 7E and Supplemental Movies 3&4).

The duration of the mitotic cell death interval was comparable between docetaxel treatment alone and co-treatment with R428 (Figure 7C). However, co-treatment caused most cells to enter mitosis and die without completing mitosis (Figure 7A). In contrast, with docetaxel alone, the death in mitosis fate occurred during the second mitosis; thus, cells had completed one mitotic event before experiencing death in mitosis (explaining why the combined cell fates total >100). To quantify this event, we recorded the timestamp of the mitotic death event, and observed a significant effect of combination treatment. Thus, the death in mitosis event occurred substantially earlier (25.14 ± 0.86 hours for combination versus 41.74 ± 1.98 hours docetaxel alone) (Figure 7D). We similarly observed enhanced mitotic death in combination-treated MDA-MB-231 cells (Figure S9D-G). Moreover, the enhanced death in mitosis was dependent on the docetaxel concentration (Figure S9D&G).
As with R428, co-treatment with MP-470, or the AXL-FC protein, and docetaxel resulted in altered cell fate during mitosis (Figure S9A,B). Similar results were seen upon co-treatment with PHA-739358 and R428 (Figure 7A). In contrast, co-treatment with R428 and gemcitabine did not alter mitosis or mitotic fate (Figure S9C). These findings support the specificity of AXL inhibition in potentiating the mitotic fate effect of anti-mitotic agents in mesenchymal tumor cells.

**AXL’s role in mitotic fate is associated with CDC2 dysregulation**

Cell death during mitosis is caspase-dependent (30), and mitotic fate is determined by both caspase activation and cyclin B1 degradation. Enhanced caspase activation and slower cyclin B1 decay promotes death in mitosis, whereas, delay in caspase activation and enhanced cyclin B1 degradation causes mitotic slippage. Docetaxel induced caspase-3/7 activation, however, upon R428 co-treatment, caspase-3/7 activity was induced faster and more robustly than with docetaxel (Figure 7F), paclitaxel (Figure S8D) or PHA-739358 (Figure S8E) alone. In contrast, no effects on cyclin B1 were observed with docetaxel, even in combination with R428 (data not shown). Co-treatment with R428 and docetaxel increased G2-M arrest from 36.2% (docetaxel alone) to 51.2% (Figure 7G). We observed enhanced PARP cleavage following co-treatment with R428 and either, docetaxel, paclitaxel, or PHA-739358 (Figure 7H and Figure S8C), confirming that the observed death in mitosis event was apoptotic.
PI-3 kinase inhibition can enhance docetaxel efficacy in breast cancer models (31), and consistent with this observation, suppression of phospho-AKT and phospho-S6 by R428 were further enhanced by docetaxel (Figure S10A). A critical regulator of mitotic entry is the cyclin-dependent kinase-1 (CDC2) (32), via a regulatory phosphorylation at Tyr15 (33), and co-treatment with R428 and docetaxel enhanced and sustained CDC2 dephosphorylation (Figure 7I and S10B). Furthermore, suppression of phospho-CDC2 was observed in R428-treated xenografts (Figure S10C); however, CDC2 protein levels are not affected by AXL inhibition (Figure S11). These observations suggest that CDC2 is regulated by AXL, and upon exposure to anti-mitotic agents, cancer cells rapidly enter mitosis and remain in mitosis until the death in mitosis event, implicating CDC2 regulation in sensitization to anti-mitotic agents in mesenchymal cancer cells by AXL inhibitors.

DISCUSSION

EMT is an increasingly recognized driver of tumor progression, and accumulating evidence implicates EMT in both innate and acquired resistance to various anti-cancer agents. Our RNA-seq analysis confirmed that AXL expression is indeed widely associated with a mesenchymal phenotype, particularly within TNBCs and erlotinib-resistant NSCLCs. However, the low percentage of AXL-positive tumors in TNBC does not correlate with the in vitro findings and remains to be fully understood. Targeting AXL might therefore be an attractive therapeutic approach to overcoming resistance associated with EMT.
Our observations do not support a role for AXL inhibition in overcoming acquired drug resistance in the EMT context. This may reflect in part the fact that many of the tool compounds used in recent reports are multi-kinase inhibitors, lacking AXL selectivity (4, 17). Furthermore, there may be off-target cytotoxic effects of AXL-targeted RNAi reagents. Notably, a recently reported analysis of NSCLC cells with acquired erlotinib resistance and increased AXL levels similarly excluded a functional role for AXL (34).

Although our findings do not support a rationale for AXL inhibition in the context of acquired TKI resistance, we found that AXL inhibition may be effective in mesenchymal tumors, specifically in combination with anti-mitotic agents. In TNBC, patients typically present a significant clinical challenge, as they do not respond to the various targeted cancer therapies due to an apparent lack of RTK activation. However, TNBC patients do show some response to taxane-based chemotherapy (35), and our studies suggest that combining anti-mitotics with AXL inhibition may be an appropriate combination therapy in this disease setting.

Taxane-based therapies exert their cytotoxic effects by binding and stabilizing microtubules, resulting in cell cycle arrest in mitosis. The response to anti-mitotic agents can vary significantly among cancer cells. Thus, some cells undergo death during prolonged mitosis, while others undergo slippage and die in interphase (29). The degree of caspase activation and the level of cyclin B1 degradation can determine cell fate during mitosis (30). Several treatments reportedly enhance the
efficacy of taxane-based therapy, such as Navitoclax (Bcl-2/Bcl-xI inhibitor), and the PI-3 kinase inhibitor GDC-0941 (22, 31, 36). In these studies the enhanced efficacy of combination treatment reflected a decrease in the duration of mitosis until mitotic cell death, an enhanced rate of apoptosis, the down-regulation of cyclin D1 and pAkt, and altered kinetics of Bcl-2 family protein stabilization. AXL engages some of these pathways, particularly the PI-3K/Akt pathway (13, 14), which we found was suppressed by R428, particularly upon co-treatment with docetaxel, which may contribute to the observed synergy. Our findings also indicate that the observed synergy with AXL inhibition reflects altered kinetics of dephosphorylation of CDC2, a cell cycle-dependent kinase that governs mitotic entry. However, it remains unclear as to how AXL, relative to other RTKs that transduce signals to largely overlapping downstream effectors, specifically regulates this signaling event in the context of mitosis.

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REFERENCES


FIGURE LEGENDS

Figure 1: AXL expression correlates with a mesenchymal signature. (A) Scatter plot representing AXL RNA expression for 643 human cancer cell lines. Red line represents mean expression within each tissue type. (B) Scatter plot illustrating AXL expression in 46 breast cancer cell lines based on Estrogen-positive (ER)/Progesterone-positive (PR), and HER2 status. Differences between TNBC subtype compared to the HER2 amp (p= 0.0058), and ER+/PR- (p= 0.0081) were calculated using a Student t-test. No differences were observed between HER2 amp and ER+/PR- (NS). Black line represents the median expression within each subtype. (C) Immunoblots indicating AXL protein in triple-negative, Estrogen-positive (ER)/Progesterone-positive (PR), and HER2-amplified breast cancer cells. (D) Immunoblots indicating AXL, MER, and TYRO-3 protein in TNBC cells. We note reported controversy regarding the tissue origin of MDA-MB-435 cells, which clusters with cell lines of melanoma origin by expression profiling. Asterisks indicate cell lines with high Vimentin expression. (E) Immunoblots showing AXL and vimentin expression in NSCLC cell lines. Cell lines were arranged according to erlotinib sensitivity as IC$_{50}$ (25). #1 indicates the most erlotinib-sensitive and #46 is the least sensitive. Lanes 47 and 49 correspond to HCC827 and PC9 cell lines, respectively, treated with TGF-β for 14 days. GAPDH levels demonstrate protein normalization. Asterisks indicate cell lines with EGFR mutation.
Figure 2: AXL expression is increased upon TGF-β-induced EMT. (A) Microscopically observed morphological changes in breast cancer cell lines HCC1954 and BT474, and lung cancer cell line PC9 treated with TGF-β for 14 days. (B) Immunoblot demonstrating loss of E-Cadherin and MER and increased vimentin and AXL upon TGF-β treatment of BT474, HCC1954, and SUM149PT, and PC9 cells. (C) Bar graph illustrating enhanced invasion capacity of TGF-β-treated (day 14) HCC1954 cells. Differences between Parental and TGF-β-treated (p= 0.033) cells were calculated by Student t-test. (D) Bar graph demonstrating increased Gas6 secretion (by ELISA) in HCC1954 and PC9 cells following TGF-β-induced EMT (day 14). Differences between Parental compared to TGF-β-treated (HCC1954, p<0.0001 and PC9, p=0.0003) were calculated by Student t-test. Error bars represent the mean ± SEM. (E) Cell viability assay demonstrating the effect of lapatinib in TGF-β-treated (day 14) HCC1954 cells. IC\textsubscript{50} values for lapatinib were calculated in Prism, Parental; IC\textsubscript{50}= 0.456µM, TGF-β; IC\textsubscript{50}= 4.036µM. (F) Cell viability assay demonstrating the effect of erlotinib in TGF-β-treated (day 14) PC9 cells. IC\textsubscript{50} values for erlotinib were calculated in Prism, Parental; IC\textsubscript{50}= 0.05µM, TGF-β; IC\textsubscript{50}= 0.214µM (G) Immunoblot showing the effect of erlotinib (ERL; 50nM) and lapatinib (LAP; 1µM) on PARP cleavage and AXL expression after 72h. All Error bars represent the mean ± SEM.

Figure 3: AXL inhibition does not re-sensitize erlotinib-resistant NSCLC cells. (A) Drug matrix heat-map grid illustrating percentage inhibition and delta Bliss for R428 in combination with erlotinib in PC9 parental and mesenchymal (MES) cell
lines (Top), HCC4006 parental and erlotinib-resistant (ERL-R) cells (Middle), and HCC827 parental and erlotinib-resistant (ERL-R) cells (Bottom). Drug matrix heat-map grids correspond to one representative experiment out of three independent experiments. (B) Immunoblot demonstrating increased AXL following prolonged erlotinib treatment (ERL-R) in HCC4006 and HCC827 cells. (C) Table illustrating the sum Bliss score for erlotinib in combination with R428. (D) Cell viability assay demonstrating the effect of R428 (1µM) in combination with erlotinib in PC9 parental and mesenchymal (MES) cells (Left) and HCC827 parental and mesenchymal (MES) cells (Right). (E) Cell viability assay illustrating the effect of R428 (1µM) in combination with erlotinib in HCC4006 parental and erlotinib-resistant (ERL-R) cells (Left), and HCC827 parental and erlotinib-resistant (ERL-R) cells (Right). All error bars represent the mean ± SEM. (F) Immunoblot demonstrating decreased AXL expression following knockdown (10nM) for 72h with four different AXL siRNAs in PC9 mesenchymal cells. NTC siRNA serves as a control. (G) Immunoblot showing the effect of AXL knockdown (10nM) for 72h and in combination with erlotinib (50nM) for a further 72h on PARP cleavage and vimentin expression in PC9 parental (PAR) and mesenchymal (MES) cells. (H) Cell viability assay demonstrating the effect of AXL knockdown (20nM) for 24h and in combination with erlotinib for a further 72h in HCC4006 erlotinib-resistant (ERL-R) cells. Error bars represent the mean ± SEM.
Figure 4: AXL inhibition synergizes with docetaxel in erlotinib-resistant mesenchymal NSCLC. (A) Drug matrix heat-map grid illustrating percentage inhibition and delta Bliss for R428 in combination with docetaxel (DTX) for PC9 parental and mesenchymal (MES) cells (Top), HCC4006 parental and erlotinib-resistant (ERL-R) cell lines (Middle), and HCC827 parental and erlotinib-resistant (ERL-R) cells (Bottom). Drug matrix heat-map grids correspond to one representative experiment out of three independent experiments. (B) Table illustrating the sum Bliss score for docetaxel in combination with R428. (C) Cell viability assay demonstrating the effect of R428 (1µM) in combination with docetaxel. IC$_{50}$ values for docetaxel single agent or in combination R428 (1µM) were calculated in Prism, PC9: PAR, 2.265nM; PAR + R428, 0.535nM; MES, >300nM; MES + R428, 0.3nM. Parental (PAR), Mesenchymal (MES). (D) Cell viability assay illustrating the effect of R428 (1µM) in combination with docetaxel. IC$_{50}$ values for docetaxel single agent or in combination with R428 (1µM) were calculated in Prism, HCC4006 (Left): PAR, 0.429nm; PAR + R428, 0.223nM; ERL-R, >300nM; ERL-R + R428, 0.191nM, HCC827 (Right): PAR, 2.759nm; PAR + R428, 1.012nM; ERL-R, >300nM; ERL-R + R428, 100nM. All error bars represent the mean ± SEM.

Figure 5: AXL inhibition enhances the efficacy of anti-mitotic agents. (A) Table summarizing the sum Bliss score upon co-treatment with R428 and docetaxel, mesenchymal cell lines indicated in blue (left panel). Right-hand panel,
drug matrix heat-map grid (right) illustrating percentage inhibition and delta Bliss for HeLa (Left) and MDA-MB-231 (Right) cell lines upon co-treatment with R428 and docetaxel (DTX) (Top), PHA-739358 (Middle), and gemcitabine (Bottom). Drug matrix heat-map grids correspond to one representative experiment out of three independent experiments. (B) Plot demonstrating the correlation between AXL expression (RPKM) and sum Bliss score of the R428 and docetaxel combination. AXL-negative cell lines were removed from analysis. R^2 value equals 0.5044 and was calculated using Pearson correlation coefficient. (C) Cell viability assay demonstrating the effect of R428 (1µM) in combination with docetaxel (Left), PHA-739358 (Middle) and gemcitabine (Right). Error bars represent the mean ± SEM. MDA-MB-231 IC_{50} values for docetaxel single agent treatment or in combination with R428 were calculated in Prism, DMSO, 0.275nM; R428 (1µM), 0.147nM; R428 (3µM), 0.053nM. IC_{50} values for PHA-739358 single agent treatment or in combination R428 were calculated in Prism, DMSO, 1.626µM; R428 (1µM), 0.336µM; R428 (3µM), 0.17µM. (D) Syto 60 cell staining of HeLa cells treated for 72h with docetaxel (DTX; 3nM), R428 (1µM), AXL-FC (10µg/ml), or MP-470 (1µM). (E) Syto 60 cell staining of HeLa cells upon siRNA knockdown of AXL (10nM) or non-targeting control (NTC) for 72 hours and in combination with docetaxel (DTX, 1nM) for a further 72 hours, left panel. Immunoblot demonstrating AXL protein expression following knockdown in combination with docetaxel (1nM), right panel.

Figure 6: AXL inhibition in combination with docetaxel retards the growth of mesenchymal tumor cells in vivo. (A) Immunoblots showing phospho-AKT
(pAKT), total AKT, phospho-S6 (pS6) and total S6, in individual MDA-MB-231 xenograft tumors following R428 treatment. (B) Tumor growth assay showing the anti-tumor effect of R428 in combination with docetaxel in Hela xenografts. Differences between the R428 and the docetaxel combination group compared to the Vehicle group (p<0.0001), and individual treatment with R428 (p=0.0001) and docetaxel (p<0.0001) were calculated using two-way ANOVA. Error bars represent the mean ± SEM. Percentage body weight change is shown at the bottom. (C) Tumor growth assay showing the anti-tumor effect of R428 in combination with docetaxel in MDA-MB-231 xenografts. Differences between the R428 and the docetaxel combination group compared to the Vehicle group (p=0.0033), and individual treatment with R428 (<0.0001) and docetaxel (p<0.0001) compared to the combination group were calculated using two-way ANOVA. Error bars represent the mean ± SEM. Percentage body weight change is shown at the bottom.

Figure 7: Axl inhibition in combination with anti-mitotic agents promotes mitotic death. (A) Table representing the fate of 100 individual cells following exposure to R428 (1µM), docetaxel (DTX; 3nM), PHA-739358 (PHA; 100nM), or the indicated combinations based on a 72h microscopy assay. (B) Scatter plot demonstrating the duration of completed mitosis (h) for 100 individual cells upon each drug treatment: R428 (1µM), docetaxel (DTX), PHA-739358 (PHA) and combinations in HeLa cells in a 72h assay. Red line indicates Mean. (C) Scatter plot demonstrating the duration of the mitotic death interval in hours (h) of 100
individual cells upon each drug treatment: R428 (1µM), docetaxel (DTX) and the combination in HeLa cells in a 72h assay. Red line indicates Mean. (D) Scatter plot demonstrating a significant difference as assessed using Student’s t-test (HeLa p= <0.0001) of the time of mitotic death in hours (h) of 100 individual cells upon each drug treatment: R428 (1µM), docetaxel (DTX; 3nM) and the combination in HeLa cells in a 72h assay. Red line indicates Mean. (E) Microscopy images demonstrating mitosis following treatment with R428 (1µM), docetaxel (DTX; 3nM) or the combination in HeLa cells at 18h (left panel) and 36h (right panel). (F) Bar graph representing caspase-3/7 activation following treatment with R428 (1µM), docetaxel (DTX; 10nM) or the combination in HeLa cells during a 18h, 24h and 36h time course. Error bars represent the mean ± SEM. (G) Histogram plots demonstrating DNA content upon exposure of HeLa cells to R428 (1µM) in combination with docetaxel (DTX; 10nM) after 8h. (H) Immunoblot demonstrating apoptosis (cleaved PARP) in HeLa cells after PHA-739358 (PHA) or docetaxel (DTX) treatment in combination with R428 (1µM) for 72 hours. (I) Immunoblots showing phospho-CDC2 (pCDC2) and total CDC2 in HeLa cells following treatment with R428 (1µM), docetaxel (DTX; 3nM) or the combination (COMBO) during an 8 hour assay period.
Sensitivity to Erlotinib

**Figure 1:**

A. AXL RNA Seq data for various cancer cell lines, showing expression levels.

B. AXL RNA Seq data for breast cancer cell lines, with a significant increase in expression for ER+PR+ cell lines.

C. Western blot analysis of TGF-β and AXL expression in TNBC and ER+PR- cell lines.

D. Western blot analysis of AXL, MER, TYRO-3, and GAPDH in triple negative breast cancer cell lines.

E. Western blot analysis of AXL, Vimentin, and GAPDH in lung cancer cell lines.
Figure 2:

A. Parental and TGF-β treated HCC1954, BT474, and PC9 cells.

E-Cadherin
Vimentin
AXL
GAPDH

B. Western blot analysis of E-cadherin, Vimentin, AXL, and GAPDH in BT474 and HCC1954 cells.

C. Bar graph showing fold change in invasion for Parental and TGF-β treated HCC1954 and PC9 cells.

D. Bar graph showing Gas6 ng/ml per 1x10^6 cells for Parental and TGF-β treated HCC1954 and PC9 cells.

E. Graph showing % cell viability with increasing Lapatinib (µM) for Parental and TGF-β treated cells.

F. Graph showing % cell viability with increasing Erlotinib (µM) for Parental and TGF-β treated cells.

G. Western blot analysis of Cleaved PARP, E-Cadherin, Vimentin, AXL, and GAPDH in PC9 and HCC1954 cells.
### Figure 4

#### A

**PC9 Parental**

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**Combination with R428 and docetaxel**

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Figure 5:

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**B**

$R^2 = 0.5044$

**C**

MDA-MB-231

**E**

NTC SiRNA    Axl SiRNA

DMSO    Axl

GAPDH
Figure 6:

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B

HeLa Xenograft

C

MDA-MB-231 Xenograft
Figure 7:

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B

C

D

E

F

G

H

I

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AXL inhibition sensitizes mesenchymal cancer cells to anti-mitotic drugs

Catherine Wilson, Xiaofen Ye, Thinh Q. Pham, et al.

Cancer Res Published OnlineFirst August 14, 2014.